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Biochemical characterization of *Aspergillus niger* Cfcl, a glycoside hydrolase family 18 chitinase that releases monomers during substrate hydrolysis

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The genome of the industrially important fungus *Aspergillus niger* encodes a large number of glycoside hydrolase family 18 members annotated as chitinases. We identified one of these putative chitinases, Cfcl, as a representative of a distinct phylogenetic clade of homologous enzymes conserved in all sequenced *Aspergillus* species. Where the catalytic domain of more distantly related chitinases consists of a triosephosphate isomerase barrel in which a small additional ($\alpha + \beta$) domain is inserted, Cfcl-like proteins were found to have, in addition, a carbohydrate-binding module (CBM18) that is inserted in the ($\alpha + \beta$) domain next to the substrate-binding cleft. This unusual domain structure and sequence dissimilarity to previously characterized chitinases suggest that Cfcl has a novel activity or function different from chitinases investigated so far. Following its heterologous expression and purification, its biochemical characterization showed that Cfcl displays optimal activity at pH 4 and 55–65 °C and degrades chitin oligosaccharides by releasing *N*-acetylglucosamine from the reducing end, possibly via a processive mechanism. This is the first fungal family 18 exochitinase described, to our knowledge, that exclusively releases monomers. The *cfcI* expression profile suggests that its physiological function is important in processes that take place during the late stages of the *aspergillus* life cycle, such as autolysis or sporulation.

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INTRODUCTION

Aspergillus niger is a filamentous ascomycete of significant industrial importance. This fungus is the main producer of citric acid and is used as an expression host for both heterologously and homologously (over)expressed extracellular enzymes (Schuster *et al.*, 2002). The genome sequences of two *A. niger* strains each revealed the presence of 16 genes encoding putative chitin-degrading enzymes (Andersen *et al.*, 2011; Pel *et al.*, 2007). Chitinolytic enzymes are of major physiological importance in their fungal hosts. They are involved in the degradation of chitin substrates (Lopez-Mondéjar *et al.*, 2009) and may become expressed during mycoparasitism as discussed by Seidl (2008). In addition, chitinases are thought to modify chitin present as a structural component in the fungal cell wall

and thereby play a role during morphological changes such as autolysis, hyphal branching or germination of conidia (Shin *et al.*, 2009; Yamazaki *et al.*, 2008).

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology distinguishes two chitinolytic activities (IUBMB, 1992). The β -*N*-acetylhexosaminidases (EC 3.2.1.52) release *N*-acetyl-D-hexosamine residues from the non-reducing terminus of *N*-acetyl- β -D-hexosaminides, such as chitin oligosaccharides. All fungal enzymes with this activity belong to family 20 of the sequence- and fold-based glycoside hydrolase (GH) families, as described in the CAZy database (Cantarel *et al.*, 2009). Chitinases (EC 3.2.1.14) randomly hydrolyse β (1,4) linkages between *N*-acetyl- β -D-glucosamines in chitin. Fungal chitinases belong to GH18. Meanwhile, chitinolytic enzymes have been identified with a substrate/product specificity that is not covered by this nomenclature. Chitobiosidase refers to chitinases releasing mainly or exclusively chitobiose, a common activity in family GH18. In addition, the terms endochitinase and exochitinase are generally applied to distinguish between enzymes that randomly cleave chitin substrates and enzymes that release products from either end of their substrates, respectively (Seidl, 2008). In this

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Abbreviations: CBM, carbohydrate-binding module; GH, glycoside hydrolase; GlcNAc, *N*-acetyl-D-glucosamine; GPI, glycosylphosphatidylinositol; HPAEC, high performance anion-exchange chromatography; LysM, lysine motif; MBP, maltose-binding protein; PAD, pulsed amperometric detection; pNP, 4-nitrophenol; TIM, triosephosphate isomerase.

paper the term exochitinase is used for enzymes releasing either monomers or chitobiose from the substrate end.

The genomes of filamentous Ascomycota usually contain 10–20 genes encoding GH18 enzymes (Karlsson & Stenlid, 2008; Seidl *et al.*, 2005). GH18 chitinases use substrate-assisted catalysis which involves the *N*-acetyl group of chitin and they retain the chitin β -anomeric configuration in their products (van Aalten *et al.*, 2001). After recent revision of the phylogenetic groups harbouring fungal GH18 members, three groups have been distinguished (Karlsson & Stenlid, 2008; Seidl *et al.*, 2005). Group B (previously class III) members display sequence similarity to plant chitinases. Their active site, on the top of a triosephosphate isomerase (TIM) barrel, forms an open groove (Rush *et al.*, 2010; Terwisscha van Scheltinga *et al.*, 1994). Chitinases with such an active site show endo-activity (Hoell *et al.*, 2005; Horn *et al.*, 2006; Terwisscha van Scheltinga *et al.*, 1994). Group A enzymes (previously class V) have sequence similarity to bacterial chitinases. Their catalytic domain resembles that of group B, but an additional domain is inserted in the TIM barrel between β -sheet 7 and α -helix 7 (Perrakis *et al.*, 1994). This 70–90 amino acid ($\alpha + \beta$) domain forms part of the wall of the deep substrate-binding cleft and influences substrate and product specificities (Li & Greene, 2010; Zees *et al.*, 2009). Chitinases of group A have exo- and/or processive activity (Fukamizo *et al.*, 2001; Jaques *et al.*, 2003). Group C members were identified recently, when the availability of complete genome sequences of filamentous Ascomycota revealed chitinase sequences distinct from, but with sequence similarity to, the known group A proteins (Karlsson & Stenlid, 2008; Seidl *et al.*, 2005). The large proteins (up to 200 kDa) often contain multiple carbohydrate-binding modules (CBMs) of family 18 (CBM18) and lysine motif (LysM) modules (Gruber *et al.*, 2011). CBM18 modules consist of around 40 amino acids, organized in a structure of small α -helices, β -sheets and mostly coil regions, for which binding to chitin and chitin oligosaccharides has been shown (Boraston *et al.*, 2004).

Analysis of expression conditions showed that CfcI of *A. niger* N402 is detected after the exponential growth phase, during nutrient limitation (Adav *et al.*, 2010; Jørgensen *et al.*, 2010; Lu *et al.*, 2010; Martens-Uzunova & Schaap, 2009; van den Berg *et al.*, 2010; Yuan *et al.*, 2008). It is therefore an interesting candidate for a chitinase involved in sporulation or autolysis, the process where the ageing mycelium degrades part of its own hyphae (White *et al.*, 2002). Our first phylogenetic analysis of GH18 members in all eight sequenced *Aspergillus* species indicated that CfcI of *A. niger* N402 represents a conserved phylogenetic clade. Members of this clade show sequence similarity to both group A and C chitinases and they have a unique domain structure where a CBM18 is inserted in the catalytic domain. This paper provides the first description of the characterization of an enzyme from this phylogenetic clade. Biochemical characterization of the heterologously expressed and purified protein showed that CfcI catalyses the release

of *N*-acetyl-D-glucosamine (GlcNAc) from the reducing end of chitin oligosaccharides. To our knowledge, this is the first report of a fungal GH18 chitinase with this activity.

METHODS

Phylogenetic analysis. *A. niger* CBS 513.88 (Pel *et al.*, 2007) amino acid sequences containing Pfam motif PF00704 and catalytic residue motif PS01095 were used to perform multispecies BLAST against the *Aspergillus* Genome Database (AspGD, <http://www.aspergillusgenome.org>) to identify all GH18 members in each genome (Arnaud *et al.*, 2010). Sequences were aligned with M-Coffee (Moretti *et al.*, 2007) to identify group members. Separate alignments were made for both B and A/C groups using MUSCLE (Edgar, 2004). Alignments were trimmed to the GH18 catalytic domain using BioEdit (Hall, 1999). MEGA4 (Tamura *et al.*, 2007) was used to generate bootstrapped (5000 replicates) neighbour-joining phylogenetic trees, using the Poisson correction model with pairwise gap deletion. *A. niger* An09g06400 (group B) and An02g07020 (group A) were used as out-groups to root the neighbour-joining trees of groups A and C, and group B, respectively.

Protein sequence analysis. Conserved motifs were identified by using InterProScan (Zdobnov & Apweiler, 2001) and ScanProsite (Gasteiger *et al.*, 2003), signal peptides by using SignalP (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997) and glycosylphosphatidylinositol (GPI) anchors by using Big PI fungal predictor (Eisenhaber *et al.*, 2004). Sequence similarities were calculated with MEGA4, using the p-distance model (Tamura *et al.*, 2007).

Construction of the *Escherichia coli* expression vector pBAD-MBP-21. Standard DNA manipulation techniques were used (Sambrook *et al.*, 1989) and DNA sequences obtained by PCR were confirmed through sequencing. RNA isolated from a batch culture of *A. niger* N402 grown for ~90 h in a 5 l bioreactor (BioFlo 3000, New Brunswick Scientific) in minimal medium (Bennett *et al.*, 1991) with 0.8 % maltose was kindly provided by B. Nitsche and A. Ram of the University of Leiden, The Netherlands. RNA was converted to cDNA using the transcriptor high fidelity cDNA synthesis kit (Invitrogen) with the provided anchored-oligo(dT)₁₈ primer. The coding region of *cfcI* was amplified using high fidelity PCR enzyme mix (Fermentas) and gene-specific primers Fw15 5'-GATAAGTCGAATTCATGAGC-CTGCAGTGCCTGGC-3' (*EcoRI* site underlined) and Rev21 5'-GGAGGGTGACCAAGCTTTCAATGATGATGATGATGATGCCTG-GCAGCAACACCCTC-3' (*HindIII* site underlined, stop codon in bold and 6 × His tag in italics) under the following conditions: 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s, followed by 7 min elongation. The amplified fragment was cloned into *EcoRI*/*HindIII*-digested pBAD-MBP (Heuts *et al.*, 2007), to produce pBAD-MBP-21 which encodes the 89.7 kDa CfcI with an N-terminal maltose binding protein (MBP) and a C-terminal 6 × His tag fusion.

Construction of *E. coli* expression vector pET-15b-25 and *Pichia pastoris* GS115 harbouring pPICZ α -A-21. To investigate whether the observed characteristics of the MBP-CfcI fusion protein were dependent on the presence of MBP or lack of glycosylation, CfcI was produced both without the MBP fusion domain in *E. coli* and as a secreted glycoprotein in *P. pastoris*. The *cfcI* gene was amplified from pBAD-MBP-21 using Fw25 (5'-GATCGACATATGCGGTTGCC-ATGTACGTTGATG-3', *NdeI* site underlined) and Rev25 (5'-GATCGAGGATCCCTACCTGGCAGCAACACC-3', *BamHI* site underlined, stop codon in bold). Ligation into *NdeI*/*BamHI*-digested pET-15b resulted in pET-15b-25, which allowed the expression of CfcI with an N-terminal 6 × His tag, with a predicted molecular mass of

47.3 kDa. To construct pPICZ α -A-21 for CfcI expression in *P. pastoris*, the *cfcI*-coding region was obtained as an *EcoRI*/*Sall* fragment from pBAD-MBP-21 and ligated into pPICZ α -A. The construction of a strain GS115 derivative, harbouring pPICZ α -A-21, was performed using the EasySelect *Pichia* expression kit (Invitrogen) according to the manufacturer's instructions.

Protein expression and purification. Precultures of *E. coli* TOP10 (Invitrogen) harbouring pBAD-MBP-21 were diluted 1:100 in Luria Broth (Sambrook *et al.*, 1989). After growth at 30 °C until OD₆₀₀ ~0.4, expression was induced with 0.1% (w/v) arabinose. After 4 h, cells were harvested by centrifugation, resuspended in 20 mM Tris/HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA and broken by sonication. CfcI-MBP was partly purified by affinity chromatography using amylose resin (New England Biolabs) and anion-exchange chromatography at pH 8 using a HiTrap Q Sepharose HP column (1 ml, GE Healthcare). Progress in purification was followed by SDS-PAGE and the purity in a representative final preparation was analysed by identification of proteins by LC-MS/MS, kindly performed by DSM Biotechnology Centre, Delft, The Netherlands. Protein concentrations were determined with Bradford reagent (Bradford, 1976), using BSA as a standard. Unless stated otherwise, all experiments were performed using the CfcI-MBP fusion protein, referred to as CfcI-MBP.

CfcI without MBP was obtained using *E. coli* BL21 DE3 harbouring pET-15b-25, essentially as described above. Cells were grown at 18 °C, induction was performed with 25–200 μ M IPTG and purification was performed using Ni-NTA resin affinity chromatography. CfcI was obtained as a secreted glycoprotein from *P. pastoris* strain GS115-pPICZ α -A-21, which was grown for 48 h in a buffered complex glycerol medium, followed by 48 h growth in a buffered complex methanol-induction medium, using the EasySelect *Pichia* expression kit (Invitrogen) according to the manufacturer's instructions. CfcI was partly purified by anion-exchange chromatography. N-Glycosylation was verified by incubation of denatured CfcI with N-glycosidase F (New England Biolabs) and subsequent visualization was carried out by SDS-PAGE of the protein molecular mass reduction.

Enzyme activity assays. Chitinase activity was routinely measured by the 4-nitrophenol group (pNP) release in incubations of 60 μ l containing 50 mM sodium acetate buffer pH 5, with 1 mM (GlcNAc)₃-pNP or 0.5 mM (GlcNAc)₂-pNP at 45 °C. Samples of 5 μ l were taken at set intervals and the reaction was stopped by addition to 2.5 μ l 1 M NaCO₃. The A₄₀₅ was determined using a Nanodrop ND-1000 spectrophotometer. The initial rate ($\Delta A_{405} \text{ min}^{-1}$) is represented by the slope of the trend line produced by linear regression applied to each dataset. The protein concentration was adapted to obtain at least five data points. Measurements were performed in triplicate and expressed as mean \pm SD. Data were converted to nmol pNP min⁻¹ (mg protein)⁻¹ using a reference 4-pNP solution (Sigma-Aldrich). One unit of enzyme activity (U) was defined as the amount of activity needed to release 1 μ mol pNP min⁻¹ from 1 mM (GlcNAc)₃-pNP at 45 °C, pH 5.

Determination of kinetic parameters. Kinetic parameters were determined at 45 °C in 450 μ l incubations containing 50 mM sodium acetate buffer pH 5 and 0.1–2.5 mM (GlcNAc)₂-pNP or 0.05–1.2 mM (GlcNAc)₃-pNP. Reactions were started by the addition of 9.3 or 2.5 mU CfcI-MBP. At 1, 2, 3 and 4 min, 100 μ l samples were inactivated in 100 μ l 1 M NaCO₃. The initial rate was calculated as described above, based on at least three data points with measurements performed at least in duplicate. The production of pNP was linear in time under the assay conditions and a maximum of 10% of the available substrate was used. Rates obtained from two independent experiments were fitted to the

Michaelis–Menten equation using SigmaPlot 12.0. Resulting values are given as means \pm SEM.

Determination of reaction parameters. The pH optimum was determined by assaying activity on (GlcNAc)₂-pNP and (GlcNAc)₃-pNP at pH 3–6 in 50 mM sodium citrate buffer and at pH 6–8 in 50 mM sodium phosphate buffer at 45 °C. The temperature optimum was determined by measuring activity on (GlcNAc)₃-pNP in 50 mM sodium acetate buffer pH 5, at temperatures from 25 to 75 °C. Temperature stability was determined by incubating either 0.9 μ g CfcI-MBP μ l⁻¹ or 0.11 μ g CfcI glycoprotein μ l⁻¹ at 30–60 °C at pH 5 and assaying remaining activity with (GlcNAc)₃-pNP at 45 °C, pH 5.

Determination of substrate range. CfcI-MBP (0.5–2 mU) was incubated with 10 μ M–1 mM chitin oligosaccharide, with 0.01–0.1% (w/v) chitin or chitosan or fungal cell wall material (see below) in sodium acetate buffer, pH 5, at 45 °C. Product formation was detected either by MALDI-TOF-MS, using 2,5-dihydroxybenzoic acid matrix in an Axima Performance (Shimadzu Biotech), with high performance anion-exchange chromatography (HPAEC) as described below or by the release of pNP. Substrates (GlcNAc)_(1–6), (glucosamine)_(1–6) and pNP-labelled (GlcNAc)_(1–3) were obtained from various suppliers (Carbosynth, Sigma-Aldrich, Megazyme and Acros). Chitin and chitosan ($\geq 75\%$ deacetylated), purified from shrimp shells, were from Sigma-Aldrich. Cell walls were isolated from *A. niger* strain N402 (Bos *et al.*, 1988), which was grown for 48 h at 30 °C, 180 r.p.m., in minimal medium (Bennett *et al.*, 1991) supplemented with 0.5% (w/v) yeast extract and 0.1% (w/v) Casamino acids. The mycelium was harvested by filtration over miracloth (Calbiochem), washed with ultrapure water and broken by sonication in 50 mM sodium acetate buffer, pH 5. Cell walls were harvested by centrifugation (5900 g for 10 min), washed three times and boiled for 10 min to remove cell-wall-associated enzymic activities. The cell wall was fractionated into an alkaline-soluble and alkaline-insoluble fractions, essentially as described by Fontaine *et al.* (2000).

HPAEC-pulsed amperometric detection (PAD) analysis of reaction time course. A quantitative HPAEC assay with PAD was used to follow the reaction time course. Substrate hydrolysis and product formation by CfcI-MBP on (GlcNAc)_(3–6) oligosaccharides was followed by sampling reactions of 60 μ l containing ~1 mM substrate, 50 mM sodium acetate buffer pH 5 and 1.44 mU CfcI-MBP activity at 45 °C. The reaction was terminated by dilution of the sample in 245 μ l ultrapure water preheated to 90 °C. Analysis was performed on an ICS-3000 system equipped with a CarboPac PA-1 analytical and guard column (Dionex).

Anomeric configuration of reaction products. To identify the anomeric configuration of CfcI-MBP products, 2.9 mU CfcI-MBP was incubated for 2 min in 60 μ l with 0.25 mM (GlcNAc)₄ and 50 mM sodium acetate buffer pH 5 at 45 °C. Samples were analysed by HPLC immediately after incubation to prevent conversion between the anomeric forms. Volumes of 25 μ l were injected onto a TSK gel Amide 80 column (4.6 mm diameter, 5 μ m particle size, Tosch), separating oligosaccharides by the degree of polymerization and anomeric configuration (Fukamizo *et al.*, 2001). Carbohydrates were detected by UV absorption at 200 nm using a SpectraSYSTEM UV6000LP detector.

CfcI reaction specificity. To establish whether CfcI-MBP releases products from the reducing or non-reducing end, hydrolysis products of (GlcNAc)₂-pNP and (GlcNAc)₃-pNP were identified by HPAEC. Incubations contained 1 mM substrate, 25 mM sodium acetate buffer pH 5 and 1.44 mU CfcI-MBP in 60 μ l. The reaction was stopped by adding 10 μ l sample to 190 μ l 10 mM HCl. In addition, degradation of a (GlcNAc)₅ alditol was analysed. (GlcNAc)₅ (1 mg) was reduced (Hreggvidsson *et al.*, 2011) and desalted using a Carbohydrate

Ultra-Clean solid phase extraction column (Grace). Complete reduction was confirmed with MALDI-TOF-MS. (GlcNAc)₅ alditol (500 μ M) was incubated with 4 mU CfcI-MBP in 60 μ l containing 10 mM sodium acetate buffer pH 5 for 40 min, or 50 μ M (GlcNAc)₅ alditol was incubated with 20 mU CfcI-MBP in 100 μ l for 15 h. Reactions were stopped by heating and analysed by HPAEC.

RESULTS

Phylogenetic and sequence analysis

To gain insight into the diversity of aspergillus chitinases and their phylogenetic relationships, 166 GH18 protein sequences were analysed that originated from the eight sequenced *Aspergillus* species (ten strains): *A. clavatus* NRRL 1, *A. flavus* NRRL 3557, *A. fumigatus* A1163, *A. fumigatus* Af293, *A. nidulans* FGSC A4, *A. niger* ATCC 1015, *A. niger* CBS 513.88, *A. oryzae* RIB40, *A. terreus* NIH2624 and *Neosartorya fischeri* NRRL 181. The sequences form three groups: group A (previously class V), group B (previously class III) and group C. The average sequence identity within group A, B and C is 42.7, 36.9 and 40.0 %, respectively. The overall sequence identity between group A and C is 25.1 %, between group A and B is 17.7 %, and between group C and B is 17.2 %. Group B and groups A/C together form two distinct groups which show too much sequence dissimilarity to construct a reliable alignment. For these two groups, sequence alignments and phylogenetic trees were constructed separately. Each group consisted of several subgroups (Fig. 1) which were coherent with the subgroups described by Karlsson & Stenlid (2008, 2009) and by Seidl *et al.* (2005).

In general, fungal sequences of group B are divided into five subgroups, of which only B-I, B-II and B-V contain aspergillus sequences (Fig. 1a). For each analysed species, three to six proteins are found in group B. Characterized proteins are confined to subgroup B-I. *A. nidulans* ChiA (AN8241) is a GPI-anchored enzyme localized to the cell wall at hyphal tips and sites where hyphae branch (Yamazaki *et al.*, 2008). The crystal structure of *A. fumigatus* ChiA1 chitinase shows an open active site architecture, consistent with endochitinase activity (Rush *et al.*, 2010). The catalytic domains (Pfam motif PF00704, aa residues 28–323) of the ChiA orthologues share 71.3 % identity. In subgroup B-II, a clade (indicated in Fig. 1a) is found that contains *A. oryzae* AO090020000231, *A. clavatus* ACLA_006770 and two proteins of *A. fumigatus*, all of which have a C-terminal CBM19 (Pfam PF03427). Reannotation of the *A. flavus* and *N. fischeri* enzymes in this clade revealed that these proteins also contain a CBM19. Recently it was shown for the first time that family GH18 contains fungal endo- β -N-acetylglucosaminidases (EC 3.2.1.96), enzymes involved in protein deglycosylation (Hamaguchi *et al.*, 2010; Stals *et al.*, 2010); aspergillus B-V members show homology to these *Hypocrea jecorina* and *Flammulina velutipes* enzymes.

The number of group C chitinases ranges from 4 for *A. niger* CBS 513.88 to 12 for *A. terreus* NIH2624. Subdivision of these proteins in subgroups C-I and C-II based on their

catalytic domain coincides with the presence of a LysM module in some C-II proteins, which is in line with observations from others (Gruber *et al.*, 2011). The genome of *A. clavatus* does not encode subgroup C-II members. None of the putative chitinases in group C have been biochemically characterized. An14g07420 (group C-I), gw1.4.291.1 (C-I), An12g05330 (C-II) and gw1.7.232.1 (A-V) were found to be present in only one of the *A. niger* strains.

The aspergillus group A sequences are confined to subgroups A-II, A-IV and A-V (Fig. 1b). Subgroup A-V enzymes generally consist of a catalytic domain with or without a secretion signal peptide. Subgroup A-V contains a clade of orthologous proteins, indicated in Fig. 1(b), that includes ChiB of *A. nidulans*, a chitinase involved in autolysis (Yamazaki *et al.*, 2007). The catalytic domains of ChiB orthologues share 76.1 % sequence identity. The slightly more distantly related *A. fumigatus* ChiB1, shown in Fig. 1(b), has been characterized as a chitobiosidase (Jaques *et al.*, 2003).

The position of subgroup A-II in the phylogenetic tree is ambiguous; subgroup members display similarity to both group A and group C. In our analysis the A-II subgroup is located closest to the C-I/C-II subgroups, but with a relatively low bootstrap value supporting this branch. Opinions vary about whether the A-II group is most closely related to either the C-I/C-II subgroups (Karlsson & Stenlid, 2009) or the A-IV/A-V subgroups (Alcazar-Fuoli *et al.*, 2011; Karlsson & Stenlid, 2008; Seidl *et al.*, 2005). *A. niger* N402 CfcI is a representative member of the clade in subgroup A-II, which is conserved in *Aspergillus* species. Proteins in this clade have a domain structure that is atypical for chitinases; a CBM18 (Pfam PF00187) is integrated in the ($\alpha + \beta$) domain in the C-terminal region of the catalytic core. This integration site suggests that, spatially, the CBM18 is located close to the substrate-binding cleft. Two strains, *A. fumigatus* Af293 and *N. fischeri* NRRL 181, each harbour two sequences belonging to the clade. The *afu1g00310* gene has a stop codon located within CBM18, suggesting protein truncation. In addition to the CfcI clade, subgroup A-II contains AN0221, which lacks a CBM18 and has an endoplasmic-reticulum-targeting signal. *A. nidulans* harbours the only member of this second clade among the *Aspergillus* species but homologues are present in other fungi (Karlsson & Stenlid, 2009).

Both the unusual domain organization of CfcI and its orthologues, and their sequence dissimilarity to previously characterized chitinases, suggest that these enzymes may have an activity or function different from proteins investigated so far. To characterize CfcI, the protein was heterologously expressed and biochemically characterized.

Cloning and expression of CfcI

The coding region of *cfcI*, excluding the predicted secretion signal peptide, was amplified from cDNA. Initial tests to

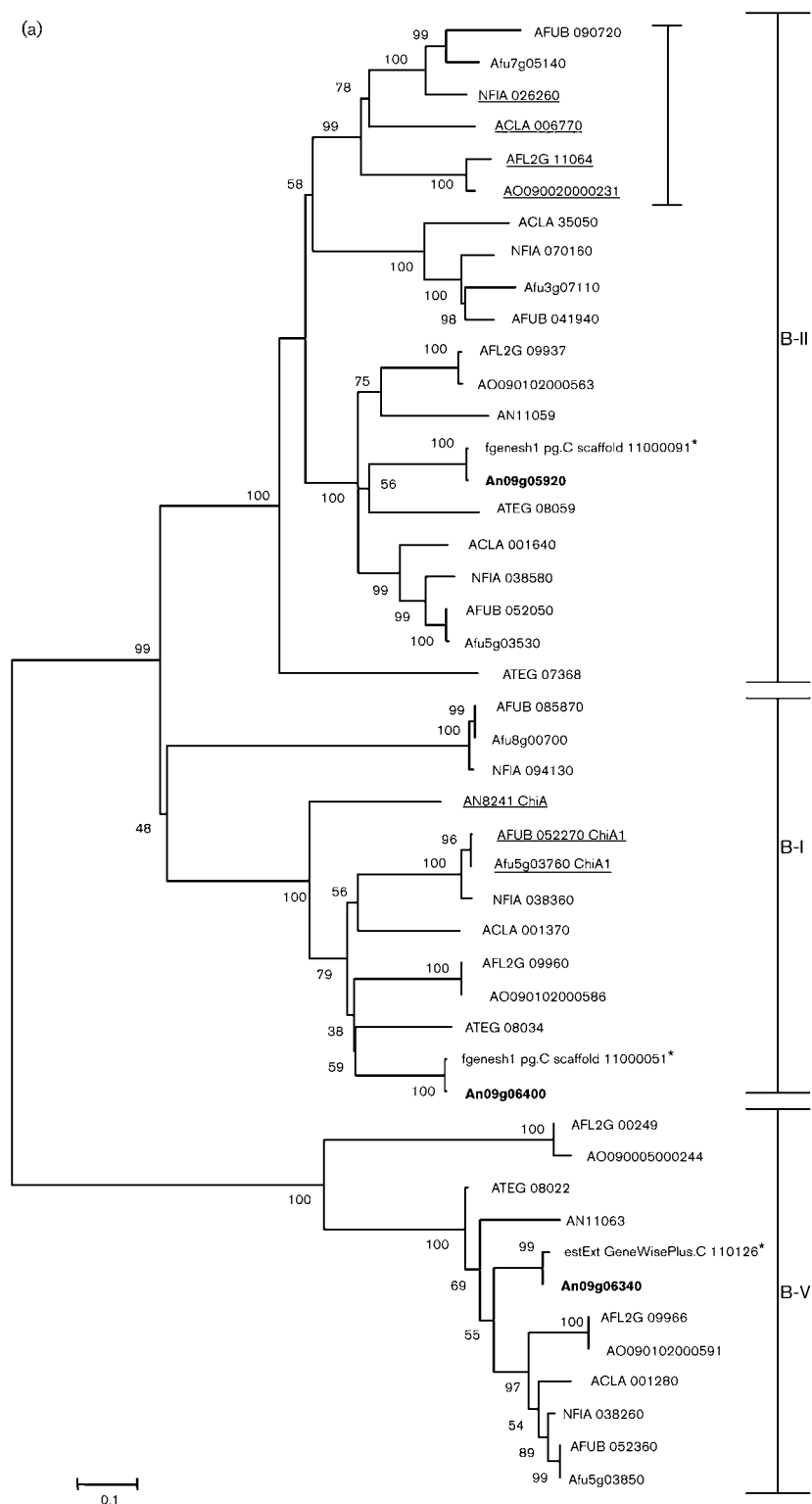
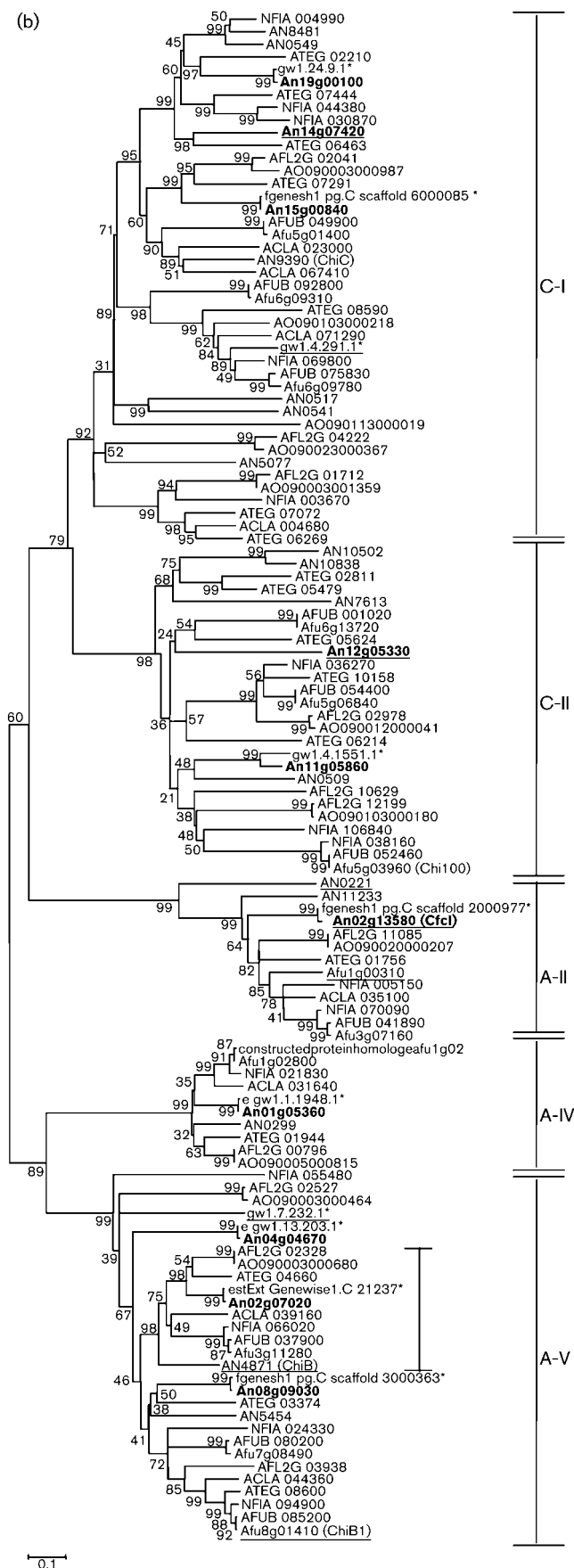


Fig. 1. Phylogenetic relationship of the 166 GH18 members present in the eight sequenced genomes of *Aspergillus* species, based on the amino acid sequences of their catalytic domains, displayed as a rooted neighbour-joining tree. Branch support is indicated by bootstrap values. Sequences are labelled with locus/protein identifiers as found in AspGD and GenBank, followed by the protein name in parentheses if gene/protein features have been characterized. Protein models labelled with their model designation only, indicated by an asterisk, belong to *A. niger* ATCC 1015. The scale bar corresponds to 0.1 amino acid substitutions per site. Subgroups are indicated by numbered bars using nomenclature described by Karlsson & Stenlid (2008). Clades discussed in the text are marked with bars; discussed sequences are underlined. *A. niger* CBS 513.88 sequences are represented in bold. (a) Relationship of group B members. (b) Relationship of group A and C members.

express CfcI resulted in insoluble protein. To facilitate folding of CfcI and thereby overproduction of soluble protein, the coding region was cloned into vector pBAD-MBP, allowing expression in *E. coli* of CfcI fused at the N terminus to a MBP (CfcI-MBP). Sequencing of the

obtained coding region showed that the position and length of introns correspond with the prediction of the recently updated gene model (GenBank accession no. XM_001400452.2). CfcI-MBP was partly purified by MBP affinity chromatography and subsequent anion-exchange



chromatography, where the bulk of the CfcI-MBP protein eluted at 300–510 mM NaCl. The fusion protein contains a C-terminal His tag, but since no binding to Ni-NTA resin could be achieved, this tag could not be used for purification. The total yield of CfcI-MBP was 1.5–2.8 mg protein (l culture⁻¹), with an activity of 0.3–0.9 mU µg⁻¹. SDS-PAGE analysis showed that CfcI-MBP was present as the main constituent of the purified protein sample, in addition to multiple bands of low intensity. LC-MS/MS analysis of the final sample of a representative purification revealed that the CfcI-MBP protein was free from contaminating proteins predicted to have interfering activities. In addition, CfcI was obtained as a secreted glycoprotein from *P. pastoris*. N-Glycosylation was verified by incubation of CfcI with N-glycosidase F. Subsequent visualization by SDS-PAGE of the protein showed a reduction in molecular mass from ~61–71 kDa to ~47 kDa, which is the predicted mass for the non-glycosylated protein.

Reaction parameters

CfcI-MBP has a relatively acidic pH optimum (pH 4–5) and activity levels drop considerably around neutral pH (Fig. 2a). Relative activity on (GlcNAc)₃-pNP at pH 6 and 7 was higher than on (GlcNAc)₂-pNP. Activity at pH 3 was observed only on (GlcNAc)₃-pNP and the enzyme was rapidly inactivated in these conditions. The maximum temperature for CfcI-MBP activity was found at 55–65 °C (Fig. 2b). Although CfcI-MBP has high activity at elevated temperatures, the enzyme was not stable under these conditions. A thermal stability assessment showed that after 30 min incubation at 40 or 50 °C, CfcI-MBP retained 75 ± 4 or 34 ± 1 % of its activity, respectively (data not shown). No residual activity was observed after incubation for 30 min at 60 °C. When repeating the experiments with a CfcI preparation obtained as heterologously expressed glycoprotein from *P. pastoris*, essentially the same pH and temperature dependencies were observed, although with higher activity at 60 and 65 °C. Also, the thermostability of the glycosylated CfcI protein was clearly increased; after 30 min incubation at 40 or 50 °C, CfcI retained 99 ± 7 or 91 ± 6 % of its activity, respectively. The *K_m* of CfcI-MBP was determined with the two commercially available pNP substrates (GlcNAc)₂-pNP and (GlcNAc)₃-pNP, giving a *K_m* of 2.0 ± 0.2 and 0.21 ± 0.02 mM, respectively. These results indicate that the affinity of CfcI-MBP for the longer substrate (GlcNAc)₃-pNP is approximately 10 times higher than for the shorter substrate (GlcNAc)₂-pNP.

Substrate range

CfcI-MBP hydrolysed native chitin oligosaccharides (GlcNAc)_(3–6), as well as (GlcNAc)₂-pNP and (GlcNAc)₃-pNP. The shorter GlcNAc-β-pNP, GlcNAc-α-pNP and (GlcNAc)₂ were not hydrolysed. Activity was observed on chitin, but this most likely resulted from degradation of the small fraction of soluble oligosaccharides present in the chitin preparation. After washing the polymer to remove

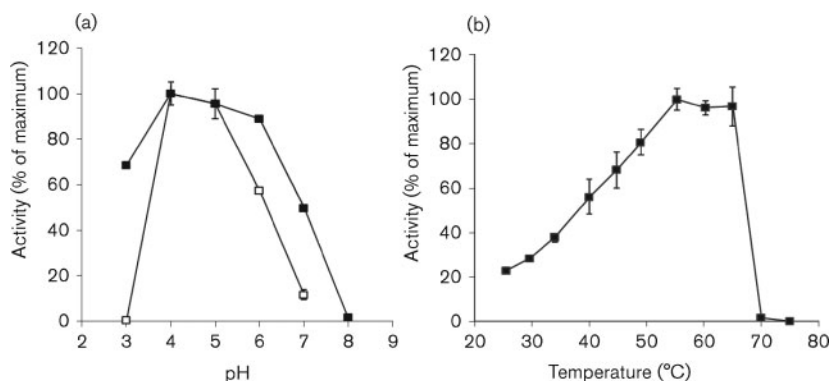


Fig. 2. Dependency of Cfcl-MBP activity on pH (a) and temperature (b). Activity was measured on (GlcNAc)₂-pNP (□) and (GlcNAc)₃-pNP (■). Values are given as percentages of the maximum activity and are the means \pm SD of three measurements. The activity of Cfcl-MBP at pH 6 in sodium citrate buffer (value not depicted) is $89 \pm 4\%$ of the activity in sodium phosphate buffer.

such oligosaccharides, a strong decrease in product formation was observed. Incubation of 10 μ g (3.2 mU) Cfcl-MBP for up to 24 h with cell walls of *A. niger* or the cell wall alkaline-insoluble fraction (containing chitin partly purified by alkaline extraction of surrounding glucans) did not result in detectable product formation.

Reaction time course

To gain an insight into the reaction specificity of Cfcl-MBP, hydrolysis of chitin oligosaccharides was followed over time by quantification of substrates and products using HPAEC (Fig. 3). (GlcNAc)₃ was degraded into equimolar amounts of GlcNAc and (GlcNAc)₂. (GlcNAc)₄ was degraded to mainly GlcNAc and (GlcNAc)₂. (GlcNAc)₃ initially accumulated, but was reutilized during the incubation, reaching a final concentration of ~ 0.2 mM. Comparable

observations were made with substrates (GlcNAc)₅ and (GlcNAc)₆; the main reaction product GlcNAc reached concentrations of 1.1 and 1.2 mM, respectively. In addition, 160–250 μ M (GlcNAc)₃ and (GlcNAc)₂ (an end product of the reaction) was produced and maxima of 45 μ M (GlcNAc)₄ and 40 μ M (GlcNAc)₅ were detected. These results demonstrate that Cfcl degrades chitin oligosaccharide substrates to predominantly produce GlcNAc. The final concentration of GlcNAc detected in each incubation corresponds with the concentration of monomers that would be generated by a strictly exo-acting enzyme that hydrolyses oligosaccharides by releasing GlcNAc from its termini until the length is decreased to (GlcNAc)₂. Similar results were obtained when repeating these experiments using Cfcl without the MBP fusion, indicating that the MBP does not affect the course of the reaction. Also, with glycosylated Cfcl obtained from

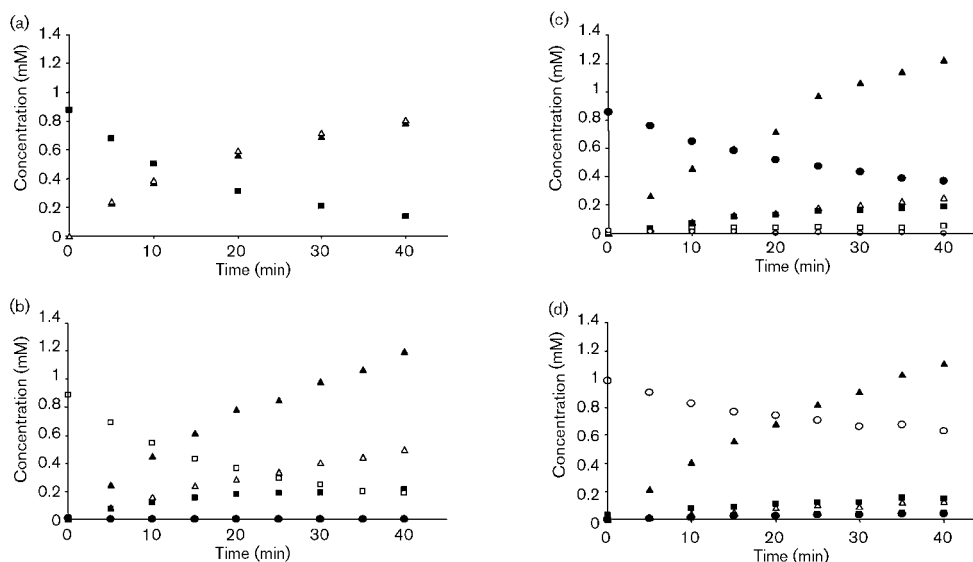


Fig. 3. Time course of substrate hydrolysis by Cfcl-MBP: (GlcNAc)₃ (a), (GlcNAc)₄ (b), (GlcNAc)₅ (c) and (GlcNAc)₆ (d). Carbohydrates, indicated as GlcNAc (▲), (GlcNAc)₂ (△), (GlcNAc)₃ (■), (GlcNAc)₄ (□), (GlcNAc)₅ (●) and (GlcNAc)₆ (○), are shown as quantified by HPAEC-PAD in a representative reaction.

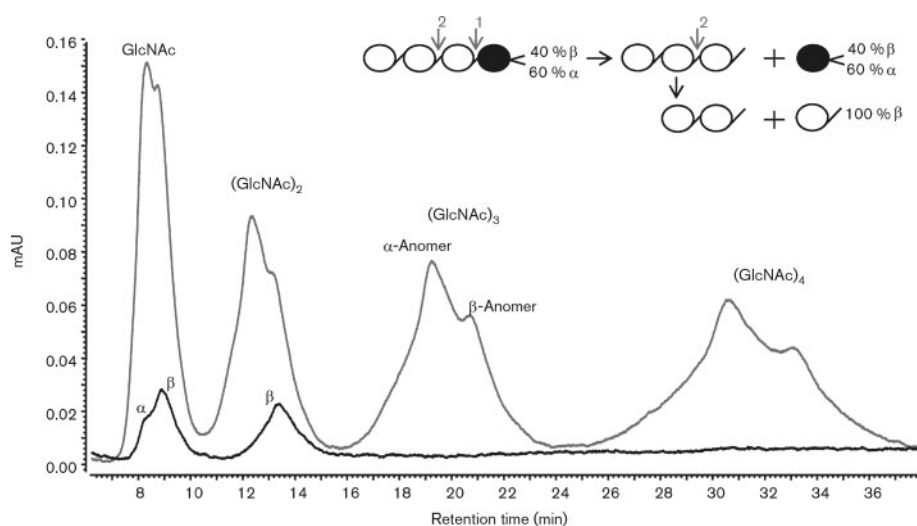


Fig. 4. HPLC analysis of the anomeric configuration of products from (GlcNAc)₄ hydrolysis by CfcI-MBP, depicted as a black line. For reference, peaks of the α and β anomer of standards of GlcNAc, (GlcNAc)₂, (GlcNAc)₃ and (GlcNAc)₄ are shown in grey. The proposed underlying mechanism of action is shown schematically. The reducing end of the substrate is indicated in black and the numbered grey arrows indicate consecutive hydrolysis events of which the products are shown.

heterologous expression in *P. pastoris*, no data were found to indicate that glycosylation affected the reaction time course (data not shown).

Anomeric configuration of reaction products and direction of substrate degradation

The anomeric forms of the CfcI-MBP products were determined by HPLC to establish whether CfcI-MBP functions with retention or inversion of the β (1,4) glycosidic linkage of the substrate. Quick, complete degradation of (GlcNAc)₄ by CfcI-MBP resulted in (GlcNAc)₂, which was present as a β -anomer [β -GlcNAc-(1,4)- β -GlcNAc], and GlcNAc, which was present predominantly as a β -anomer (Fig. 4). These results indicate that CfcI-MBP retains the β -glycosidic linkage of its substrate during hydrolysis. The anomeric forms of the reaction products also identify their origin. β -GlcNAc-(1,4)- β -GlcNAc could not originate from the reducing end of the substrate. It is a product generated by two consecutive releases of GlcNAc from the reducing end. From each (GlcNAc)₄ substrate molecule, the first cleavage results in β -GlcNAc-(1,4)- β -GlcNAc and GlcNAc in the equilibrium ratio ~60:40 α -anomer/ β -anomer. Subsequent hydrolysis releases β -GlcNAc-(1,4)- β -GlcNAc and β -GlcNAc. Together the monomers have a ~30:70 α -anomer/ β -anomer ratio, as is observed (Fig. 4) as a result of CfcI-MBP activity.

To further investigate the direction of the reaction, (GlcNAc)₂-pNP and (GlcNAc)₃-pNP hydrolysis was followed using HPAEC. (GlcNAc)₂-pNP hydrolysis exclusively resulted in the detection of (GlcNAc)₂ (Fig. 5a, note that unbound pNP cannot be detected using this method). Hydrolysis of (GlcNAc)₃-pNP yielded the detectable products (GlcNAc)₃,

(GlcNAc)₂ and GlcNAc (Fig. 5b). These products are consistent with a reaction mechanism where the substrate is degraded from the reducing end, releasing the pNP group, followed by further degradation if possible. No products were observed that would be expected to arise from CfcI-MBP action on the non-reducing end, such as GlcNAc-pNP or (GlcNAc)₂-pNP. Correspondingly, reduction of (GlcNAc)₅, which changes the reducing end of GlcNAc to an open ring structure, prevented degradation of the substrate by CfcI-MBP in conditions during which the non-modified substrate was completely hydrolysed (data not shown). Only upon prolonged incubation with high amounts of CfcI-MBP was partial substrate hydrolysis observed. Taken together, these results show that CfcI-MBP acts through a hydrolytic activity that originates at the reducing end of chitin oligosaccharides.

DISCUSSION

Although it is recognized that Ascomycetes have a large number of GH18 enzymes, the exact function of this is not yet understood (Karlsson & Stenlid, 2008; Seidl, 2008). Most likely, functional and regulatory diversification took place during evolution, although recent gene duplications may have caused redundancy. Phylogenetic analysis of GH18 enzymes showed the existence of seven groups of proteins with direct orthologues in all sequenced *Aspergillus* species. Their conservation suggests that these enzymes perform key physiological functions. So far, characterized proteins show diverse functions and locations for representatives of each group (Hamaguchi *et al.*, 2010; Jaques *et al.*, 2003; Stals *et al.*, 2010; Yamazaki *et al.*, 2007, 2008). CfcI represents one of the conserved groups of orthologues

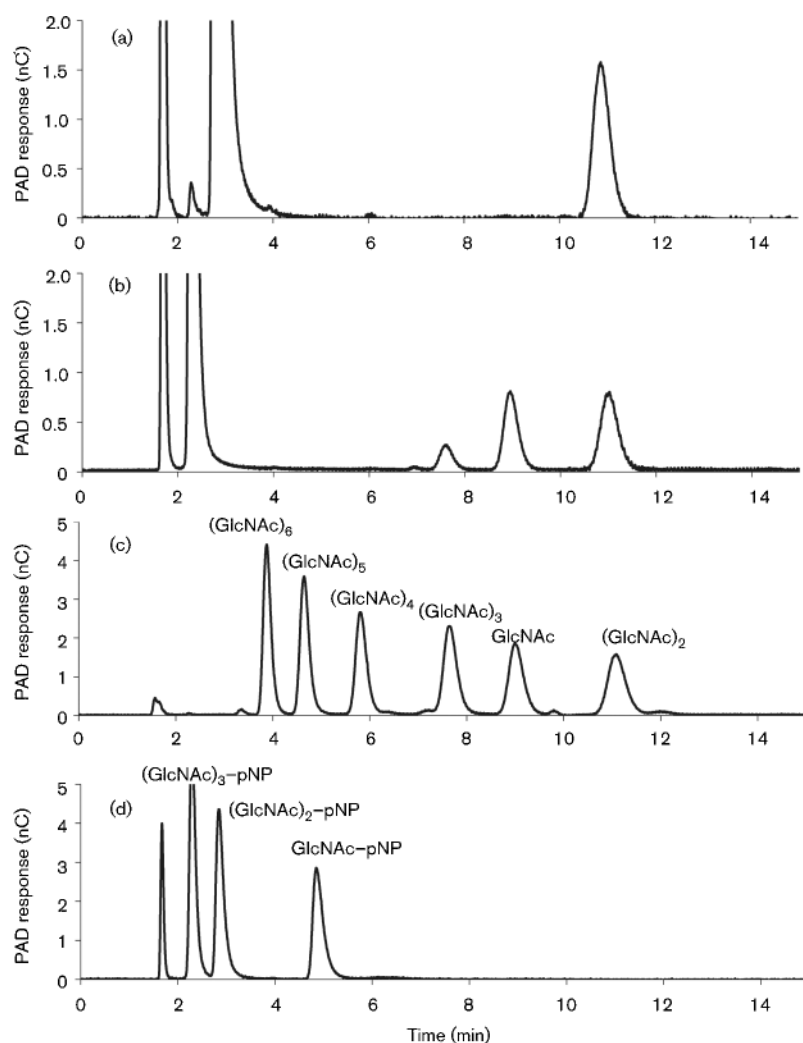


Fig. 5. Products formed by CfcI-MBP during hydrolysis of labelled substrates, (GlcNAc)₂-pNP (a) and (GlcNAc)₃-pNP (b), separated and detected by HPAEC-PAD as described in Methods. Identity of the peaks was determined by comparison to standards (GlcNAc)₍₁₋₆₎ (c) and (GlcNAc)₍₁₋₃₎-pNP (d).

of which the activity was uncharacterized until now. The atypical domain organization of CfcI suggested that the protein may have an activity or function different from known chitinases.

CfcI, expressed as fusion CfcI-MBP, is a chitinase with a mechanism of action that retains the $\beta(1,4)$ anomeric configuration found in its substrate, a common property of GH18 enzymes. However, the products formed by CfcI-MBP during substrate hydrolysis, and the direction of this reaction, are unusual. The time course analysis of hydrolysis of GlcNAc oligosaccharides revealed that CfcI releases monomers from its substrate. The molar concentrations of formed products were consistent only with exochitinase activity. Experiments with CfcI-MBP acting on both native and labelled substrates indicate that this enzyme acts on the reducing end. The exo-acting enzymes of family GH18 usually release mainly (GlcNAc)₂ from either the reducing end, such as ChiA of *Serratia marcescens*, or the non-reducing end, such as A. fumigatus ChiB and *S. marcescens* ChiB (Horn *et al.*, 2006; Jaques *et al.*, 2003). The exclusive release of monomers is a property usually found in the

GH20 family, but there the reaction starts at the non-reducing end. The activity of CfcI is unique for fungal enzymes and uncommon in the GH18 family, although it is not biochemically unique. Controlled degradation of *N*-glycosylated proteins releases oligosaccharides with a terminal chitobiose, which can be hydrolysed by lysosomal GH18 enzymes in some higher eukaryotes (Balducci *et al.*, 2008). The lysosomal enzyme from rat was also shown to be capable of hydrolysing (GlcNAc)₂₋₄ by releasing GlcNAc from the reducing end, but could not degrade GlcNAc-pNP (Aronson *et al.*, 1989). However, although biochemically similar, the protein location and pH activity profile suggest the function of these enzymes is not related to CfcI function.

Following substrate hydrolysis over time showed that CfcI-MBP produced mainly the reaction end products GlcNAc and (GlcNAc)₂. The detected amount of intermediate products was low directly from the start of the reaction. CfcI-MBP was found to have a higher affinity for the longer oligosaccharide, ruling out preferential hydrolysis of short intermediate products. This implies that the enzyme

acts processively, without releasing the substrate after the initial attack on the glycosidic bond. Since chitin consists of alternately orientated GlcNAc residues, processive cleavage of the glycosidic bonds is thought to be possible only by advancing the substrate chain by two monomers before the next cleavage event. Indeed, processive chitinases often release a dimer from chitin and studies using the partly deacetylated chitosan show that this substrate is advanced by two sugar residues, resulting in products consisting mainly of an even number of sugar units (Eijsink *et al.*, 2008). However, processive exochitinases analysed so far are all chitobiosidases and the situation may be different for CfcI. For CfcI to be able to release monomers by a processive mechanism, the substrate chain should be moved in such a way through the active site that hydrolysis can take place after each GlcNAc. This could be achieved by releasing the substrate after cleavage from the active site, but holding on to it with the CBM18 and thus allowing quick repeated binding and cleavage. The CBM18 in chitinases is usually clearly separated from the catalytic domain by a linker sequence. In CfcI, the CBM18 is embedded in the ($\alpha + \beta$) domain, which forms part of the substrate-binding cleft. This positions the CBM18 in close proximity to the substrate, enabling a direct function in substrate hydrolysis.

Taken together, our results show that the fungal enzyme CfcI is a representative of a phylogenetic clade conserved in *Aspergillus* species. CfcI is an exo-acting chitinase that releases monomers from the reducing end of chitin oligosaccharides, possibly using a processive mechanism. This activity is uncommon for GH18 members and so far is unique among fungal GH18 enzymes. This suggests the function of CfcI might be different from currently characterized GH18 family members. Expression of CfcI has been identified by proteome and transcriptome studies during the late-exponential or stationary growth phase and under nutrient limitation, but (with one exception) not during exponential growth (Adav *et al.*, 2010; Ferreira de Oliveira *et al.*, 2011; Jørgensen *et al.*, 2010; Lu *et al.*, 2010; Martens-Uzunova & Schaap, 2009; van den Berg *et al.*, 2010; Yuan *et al.*, 2008). These expression conditions indicate that the physiological function of CfcI is important in processes that take place during the late stages of the *aspergillus* life cycle, such as autolysis or sporulation. Alternatively, during periods of nutrient shortage, CfcI could generate directly available sugars in the form of GlcNAc by hydrolysing chitin oligosaccharides. The exact *in vivo* physiological function of CfcI is currently under investigation.

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